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(54) Title: COMPOSITIONS FOR DELAYED TREATMENT OF ISCHEMIA-RELATED NEURONAL DAMAGE

(57) Abstract

Methods and compositions for reducing neuronal damage related to an ischemic condition in a mammalian subject are described. The method includes administration of compositions of the invention to the subject, 4-24 hours after the onset of the ischemic condition. Compositions of the invention are effective to selectively bind omega conotoxin binding sites, and preferably to bind with high affinity to omega conotoxin MVIIA binding sites, and to selectively block neurotransmitter release from mammalian CNS neuronal cells. Also disclosed are novel peptide structures useful in the treatment method of the invention.

COMPOSITIONS FOR DELAYED TREATMENT OF ISCHEMIA-RELATED NEURONAL DAMAGE

Field of the Invention

The present invention relates to pharmaceutical compositions and methods for reducing neuronal damage with an ischemic condition, such as stroke, and for methods of screening test compounds for inclusion in such compositions and methods.

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prolonged focal ischemia, as caused by lodgement of a thromboembolus in a cerebral blood vessel, reduction of blood flow to a defined, focal region may be followed by reperfusion to part of the ischemic region, via collateral circulatory pathways. Ischemic cell death following focal ischemia has been reported to be complete 24 hours after the primary ischemic event (Nedergaard, 1987).

Several drug strategies have been proposed for treatment of stroke and other neuronal conditions related to ischemia, and these have been reviewed in recent articles (e.g., Goldberg, Wauquier). Anti-coagulants, such as heparin, have been examined, but with mixed results. Similarly, antivasoconstriction agents, such as flunarizine, excitatory neurotransmitter antagonists, such as MK-801 and AP7, and anti-edemic compounds have shown mixed results, with no clear benefits to outweigh a variety of side effects, including neurotoxicity or increased susceptibility to infection.

Two general classes of vasodilators have been studied for possible treatment of neuronal ischemic damage. Non-specific vasodilators, including papaverine, prostacyclin, pentoxifylline, and nitroprusside failed to demonstrate any clear benefit in reducing ischemic damage. A second general class of vasodilators includes a variety of calcium-antagonist vasodilator drugs. Verapamil and related compounds which prevent calcium entry into smooth and striated muscle appear to be effective only at high drug concentrations, where serious cardiotoxicity effects may ensue. Dihydropyridines, such as nimodipine, have produced mixed results -- some neurological improvement may be seen, but increased cerebral edema has also been observed.

respectively, and in applicants' PCT Application WO 91/079 the applicants have disclosed that omega-conotoxin peptides and related peptides which exhibit binding and N- or omega-type calcium channel inhibitory properties similar to those of omega-conotoxin peptides are useful in reducing neuronal damage related to ischemic conditions. In the above-referenced applications, all of which are incorporated herein by reference, experiments attesting to the efficacy of these compounds were conducted in accordance with standard experimental paradigms for examining neuroprotection. That is, test compounds were administered at the time of or up to 1 hour following the experimentally induced occlusion which caused the ischemic event. In the current application, the applicants show that reduction of neuronal damage can be enhanced when the N-channel blocking compound is administered between ***4-24 hours following ischemia, relative to immediate post-ischemia drug administration.

The applicants now show that compounds which are effective in reducing neuronal damage associated with ischemia are characterized by (a) relatively high affinity binding to an OCT binding site to which SNX-111 selectively binds (OCT site 1), and (b) relatively low affinity to a binding site selectively bound by OCT MVIIC (SNX-230) and OCT SVIB (SNX-183) (OCT site 2) in a synaptosomal preparation. This selective binding to OCT site 1 provides a basis for great screening test compounds in a screening method to select compounds for use in the treatment of cerebral ischemia.

Summary of the Inventi n

MVIIA binding site. Such high affinity is defined as an affinity which is at least as great as that of an omega conotoxin selected from the group consisting of MVIIA, MVIIB, GVIA, GVIIA and RVIA omega conotoxins.

In another embodiment, compounds useful in the treatment method are characterized further by their ability to selectively inhibit N-type voltage-gated calcium currents in neuronal tissue. In yet another embodiment, compounds useful in the treatment method are characterized by their ability to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, as evidenced by a specific activity. Generally, active compounds will exhibit activities within the range of activities of MVIIA, MVIIB, GVIA, GVIIA and RVIA omega conotoxins. In another embodiment, compounds are omega conotoxin compounds; in still another embodiment, such omega conotoxin compounds are selected from the group consisting of OCT MVIIA, OCT MVIIB, OCT GVIA, OCT GVIIA, OCT RVIA, and SNX-207.

In another aspect, the invention includes a method of screening compounds for use in reducing ischemia-related neuronal damage, such as produced by stroke, in a human subject.

In the screening method of the invention, test compounds are assayed for their binding affinities to OCT-MVIIA and OCT-MVVIC binding sites in neuronal tissue, to determine a selectivity ratio of binding for the MVIIA site with respect to the MVIIC site. The compound is selected if its selectivity ratio of binding for the MVIIA site is at least 100, and preferably, at least 500. More generally, a compound is selected, if its selectivity ratio of binding is at least as great as that of one of the omega conotoxins MVIIA, MVIIB, GVIA, GVIIA or RVIA.

following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Figures

Figure 1 shows primary sequences of several natural omega-conopeptides, MVIIA/SNX-111 (SEQ ID NO: 01), MVIIB/SNX-159 (SEQ ID NO: 02), GVIA/SNX-124 (SEQ ID NO: 03), GVIIA/SNX-178 (SEQ ID NO: 04), RVIA/SNX-182 (SEQ ID NO: 05), MVIID/SNX-238 (SEQ ID NO: 24), SVIA/SNX-157 (SEQ ID NO: 06), TVIA/SNX-185 (SEQ ID NO: 07), SVIB/SNX-183 (SEQ ID NO: 08), MVIIC/SNX-230 (SEQ ID NO: 21) and SNX-231 (SEQ ID NO: 22);

Figure 2 shows several analog omega-conopeptides SNX-190 (SEQ ID NO: 09), SNX-191 (SEQ ID NO: 10), SNX-193 (SEQ ID NO: 11), SNX-194 (SEQ ID NO: 12), SNX-195 (SEQ ID NO: 13), SNX-196 (SEQ ID NO: 14), SNX-197 (SEQ ID NO: 15), SNX-198 (SEQ ID NO: 16), SNX-200 (SEQ ID NO: 17), SNX-201 (SEQ ID NO: 18), SNX-202 (SEQ ID NO: 19), SNX-207 (SEQ ID NO: 20), SNX-260 (SEQ ID NO: 23), and SNX-236 (SEQ ID NO: 25) and their relationships to SNX-111 (SEQ ID NO: 01), SNX-185 (SEQ ID NO: 07) or SNX-183 (SEQ ID NO: 08);

Figure 3A shows voltage-gated calcium current traces induced by a voltage step from -100 or -80 mV to -20 mV in untreated N1E-115 neuroblastoma cells (curve a) and in neuroblastoma cells exposed to increasing concentrations of OCT MVIIA (SNX-111) (curves b-d);

Figure 3B plots the percent inhibition of peak inward calcium currents in neuroblastoma cells as a function of OCT MVIIA (SNX-111) (solid triangles) and OCT GVIA (SNX-124)(solid circles);

Figure 4A shows voltage-gated calcium current traces induced by a voltage step from -70 to -20 mV in human neuroblastoma cells (IMR-32) in the absence

Figures 9A and 9B show plots of displacement of binding of [^{125}I]SNX-111 (9A) and [^{125}I]SNX-183 (9B) by unlabeled OCT peptides SNX-111 and SNX-183 to 210 kilodalton proteins as described in the protein binding assay depicted in Figures 8A and 8B;

Figure 10A-10B are low-power micrographs of gerbil hippocampus CA1 region in animals after ischemia, and infusion of OCT MVIIA (SNX-111) (10A) or after ischemia and infusion of drug vehicle (10B);

Figures 11A-11D are higher power micrographs of cells in the drug-treated ischemic animals (11A, 11C, 11D), in animals receiving vehicle alone (11B), in animals showing complete protection by OCT against ischemic cell damage (11C); and in animals showing partial protection by OCT against ischemic cell damage (11D);

Figures 12A-12H show autoradiographs of coronal sections of rat brain to which is bound radiolabeled SNX-111 or SNX-183, as indicated, in the absence or presence of unlabeled peptide;

Figures 13A-13D show autoradiographs of sagittal sections of rat brain to which is bound radiolabeled SNX-111 or SNX-183, as indicated, in the absence or presence of unlabeled peptide;

Figure 14 shows amino acid sequences of active (groups I and II) and inactive (group III) OCT peptides and conserved peptide sequences within group I, Region a (SEQ ID NO: 26), Region b (SEQ ID NO: 27), Region c (SEQ ID NO: 28), Region d (SEQ ID NO: 29), Region e (SEQ ID NO: 28), and Region f (SEQ ID NO: 30), and within group II, Region s (SEQ ID NO: 27), Region t (SEQ ID NO: 31), Region u (SEQ ID NO: 32) and Region v (SEQ ID NO: 27);

Figure 15 shows a plot of hippocampal damage (CA1 region) as a function of dose of SNX-111

MVIIA, or alternatively, SNX-111, the latter to signify that the compound is synthetic in origin. Synthetic and naturally occurring peptides having the same sequence behave substantially identically in the assays and methods of treatment of the invention. The OCT MVIIA (SNX-111) and OCT GVIA (SNX-124) peptides also have the common names CmTx and CgTx, respectively. All of the OCT peptides have three disulfide linkages connecting cysteine residues 1 and 4, 2 and 5, and 3 and 6, as indicated for the MVIIA peptide in Figure 2.

Figure 2 shows analogs of natural OCT MVIIA, OCT TVIA, and OCT SVIB peptides which have been synthesized and tested in accordance with the invention. Standard single amino acid code letters are used in the figure; X=hydroxyproline; Nle=norleucine; NH_2 group at the C terminus indicates that the peptide is C-terminal amidated; G-OH indicates termination in an unmodified glycine residue.

A. <u>Preparation of OCT Peptides</u>

This section describes the synthesis, by solid phase methods, of several naturally occurring omega conotoxin (OCT) peptides and additional OCT peptides which are used in the present invention.

OCT peptides, such as those shown in Figures 1 and 2, can be synthesized by conventional solid phase methods, such as that detailed in U.S. Patent No. 5,051,403, and PCT patent application WO 91/079, both of which are incorporated herein by reference.

These methods are detailed in Example 1 herein, for the synthesis of exemplary OCT MVIIC/SNX-230.

II. <u>*In vitro* Properties of OCT Peptides</u>

Omega conotoxins also block a proportion of HVA calcium currents in neuronal tissue, and, in the presence of a maximally inhibitory quantity of dihydropyridine compound, effect substantially complete inhibition the remaining HVA currents in neuronal cells. These calcium currents are identified as N-type calcium currents, though recently a proposal that such currents be termed "omega" has been presented (Sher). Omega conotoxins bind to a specific population of binding sites. Dihydropyridines and other L-type channel blockers do not displace omega conotoxin binding, nor do omega conotoxins displace binding of ligands to L-channels. Unlike L-type calcium channels, omega channels are found predominantly, although not exclusively, in nervous tissue (Sher).

One suitable system for testing inhibition (blockage) of N-type or omega HVA neuronal calcium channels is an isolated cell system, such as the mouse neuroblastoma cell line, strain N1E115 or the human neuroblastoma cell line IMR32. Membrane currents are conveniently measured with the whole cell configuration of the patch clamp method, according to the procedure detailed in Example 2. Briefly, a voltage clamp protocol was performed in which the cell potential was stepped from the holding potential of about -100 mV to test potentials that ranged from -60 mV to +20 mV, and the cell was held at the holding potential for 5 seconds between pulses.

Figure 3A shows a typical inward calcium current in an N1E115 neuroblastoma cell elicited by a voltage step from -80 mV to -20 mV in the absence of OCT, as detailed in Example 2. In this, and most of the recordings shown, barium (Ba) replaced calcium (Ca)

value of less than about 1 μM in the assay detailed in Example 2.

Table 1

Inhibition of calcium currents in N1E-115 neuroblastoma cells

Compound	IC_{50}
GVIA (SNX-124)	10 nM
MVIIA (SNX-111)	100 nM
SVIB (SNX-183)	> 1 μM
SVIA (SNX-157)	>20 μM

Calcium currents were also measured in human neuroblastoma IMR32 cells, using techniques described above and in Example 2. Voltage-gated calcium currents were elicited by holding the cell(s) at -70 mV and administering a step-voltage to -10 mV. Current tracings from IMR-32 cells bathed in control medium (lower curve) and in medium containing 150 nM SNX-111 (upper curve) are shown in Figure 4A. In this experiment attenuation of voltage-gated calcium current is apparent in the presence of SNX-111 (upper curve), as shown by the decreased amplitude of the peak inward current.

Figures 4B and 4C show cumulative data from many consecutive currents, elicited at 15 second intervals as described above, in IMR-32 cells. In these plots, peak inward current recorded from each stimulus is recorded sequentially as a single data point. In the experiment illustrated in Figure 4B, addition of SNX-111 to the bathing m dium resulted in decreased peak inward currents; restoration of substantially normal

non-N type) current in hippocampal CA1 neuronal cells, as well as inhibition of high threshold calcium channel current in cerebella Purkinje neurons; as described in co-owned U.S. Patent Application 916,478 and subsequently published (Hillyard, *et al.*). Unlike OCT GIVA, OCT MVIIC additionally blocks calcium uptake in rat brain synaptosomes at low (2.5 μM) concentration. Unlike MVIIA, it also shows high potency in inhibition of phrenic nerve-mediated muscle concentrations in an isolated mouse diaphragm preparation.

B. Selective Inhibition of Norepinephrine Release

A second requisite property of neuroprotective compounds, in accordance with the invention, is the ability to specifically inhibit depolarization-evoked and calcium-dependent norepinephrine release in brain (CNS) neuronal cells, but not inhibit neurotransmitter release at a mammalian neuromuscular junction of a skeletal muscle. Inhibition of norepinephrine release in neuronal cells can be assayed in mammalian brain hippocampal slices by standard methods, such as detailed in Example 3.

Figure 5A shows effects of increasing concentrations of OCT MVIIA peptide on norepinephrine release from rat brain hippocampal slices which were first bathed in normal wash solution (open bars), then stimulation medium (solid bars). As seen, the compound produces a strong concentration-dependent inhibition of norepinephrine release in the presence, but not in the absence of stimulation medium. From the concentration-dependent inhibition data, the compound concentration effective to produce 50% inhibition of norepinephrine release (IC_{50}) is calculated.

high IC_{50} values for norepinephrine release. The SNX-202 peptide is a modification of SVIB peptide in which the Ser-Arg-Leu-Met residues at positions 9-12 in OCT MVIIA (SNX-111) are substituted for the Arg-Lys-Thr-Ser residues at the same positions in OCT SVIB (SNX-183). This modification significantly reduced the IC_{50} value for inhibition of norepinephrine release, but neuroprotective activity was not observed at a dose (2 μg ICV) at which SNX-111 generally provided neuroprotection. These modifications were also reflected in binding specificity of the compounds to OCT sites 1 and 2, as discussed in Part C, below.

Table 2

Inhibition of Norepinephrine Release by OCT Peptides

OCT Peptides	IC_{50} (nM)
GVIA (SNX-124)	0.8
MVIIA (SNX-111)	1.5
TVIA (SNX-185)	2.4
SNX-201	11
SNX-195	11
SNX-202	29
SVIB (SNX-183)	200
SNX-191	>100
SVIA (SNX-157)	>4500

In summary, pronounced neuroprotective activity is associated with an ability to inhibit norepinephrine releas with an IC_{50} value which is within the range of IC_{50} values measured for active OCT peptides MVIIA (SNX-111), GVIA (SNX-124), and

concentration is then used to calculate a B_{max}, the concentration of binding sites on the synaptosomes, and K_d following standard methods. In particular, the K_d value is the calculated concentration of peptide needed to half saturate the synaptosomal specific binding sites. Figure 6A shows the specific binding of radiolabeled OCT MVIIA (SNX-111) to rat brain synaptosomes, plotted as a function of OCT peptide concentration, and Figure 6B, the same data in Scatchard plot form. From the slope of the Scatchard plot line, a K_d binding value of 8.8 pM is obtained.

To determine the binding constant of a test compound for the MVIIA binding site, the test compound is added, at increasing concentrations to the synaptosome preparation having bound, radiolabeled OCT MVIIA. The synaptosomal material is then rapidly filtered, washed and assayed for bound radiolabel, as detailed in Example 5B. The binding constant (K_d) of the test compound is determined from computer-fit competitive binding curves, such as shown in Figure 7A for MVIIA (SNX-111) peptide, to determine first the IC_{50} value of the compound, i.e., the concentration which gives 50% displacement of labeled MVIIA peptide, then calculating K_i from the K_d value of OCT MVIIA and the IC_{50} value of the compound, as detailed in Example 5. IC_{50} values for a number of OCT peptides for inhibition of OCT MVIIA binding are shown in Table 3. The compounds are arranged in order of increasing IC_{50} values.

Table 3

Competition of ^{125}I-MVIIA (SNX-111) Binding by OCT Peptides

A number of OCT peptide compounds which were tested gave IC_{50} and K_i values lower than or within the ranges of those of OCT peptides MVIIA (SNX-111), GVIA (SNX-124), and/or TVIA (SNX-185), and these compounds should thus be considered candidates as neuroprotective compounds. However, some of these compounds, such as SNX-201, SNX-195, and SNX-202 have IC_{50} values for inhibition of norepinephrine release which are outside the range of neuroprotective compounds (Table 2), and thus these compounds do not meet all of the criteria for neuroprotective compounds.

b. Binding to OCT Site 2. It has also been discovered, in accordance with the invention, that compounds with high neuroprotective activity show relatively low binding affinity for a second OCT binding site. This site is defined by binding of radioiodinated OCT SVIB (SNX-183) or radioiodinated OCT MVIIC (SNX-230) binding to neuronal membranes. Conversely, high binding affinity for this second site is observed with some inactive compounds. IC_{50} and K_i values for compound binding to this site are calculated, as above, by determining the K_d of radioiodinated OCT SVIB (SNX-183) or of radiolabeled OCT MVIIC (SNX-230) for binding to a synaptosome preparation, then using competitive displacement of the labeled compound by the test compound, to determine the IC_{50} and K_i values of the test compound.

Saturation binding of [^{125}I]-SNX-230 to a rat brain synaptosomal preparation showed that the radiolabeled SNX-230 also displayed high affinity (apparent K_d = 0.012nM) but that the ranked order of potencies for displacement of this binding by the same set of OCT peptides is very different from that

properties between neuroprotective compounds, and those which show no neuroprotective activity within the range of concentrations tested.

Table 4

Selectivity of Conopeptides for Site 1 and Site 2

Compound	Ki (nM) for competition[a] with:		Selectivity[b] for:	
	[^{125}I]-SNX-111	[^{125}I]-SNX-230	site 1	site 2
SNX-111	0.003	200	67,000	: 1
SNX-124[c]	0.009	315	35,000	: 1
SNX-157	500	>100,000		
SNX-159	0.03	14	470	: 1
SNX-178	1.3	ND[d]		
SNX-182	0.4	140	350	: 1
SNX-183	0.3	4	13	: 1
SNX-185	0.08	3,000	38,000	: 1
SNX-230	0.17	.02	1	: 8.5

[a]Ki values were derived from analysis of competitive binding performed as described in Example 5.

[b]Selectivity is expressed as the ratio of the Ki value for competition with [^{125}i]-SNX_230 (MVIIC) vs. the Ki value for competition with [^{125}I]-SNX-111 (MVIIA).

[c]The result for SNX-124 (GVIA) is an apparent Ki under the given experimental conditions. It is not possible to calculate a true Ki value since GVIA binds to its receptor irreversibly.

[d] = not determined.

From the foregoing, it is seen that neuroprotective compounds in accordance with the invention are characterized by a high binding affinity for the MVIIA binding site, OCT site 1, on neuronal membranes. The binding affinity for this

peptide SNX-201 is identical to SNX-111 except for residues 9-12 (-S-R-L-M-) which have been replaced by the corresponding residues (-R-K-T-S-) from SNX-183. Similarly, SNX-202 contains residues 9-12 from SNX-111 in place of the corresponding residues in the sequence of SNX-183.

Replacement of residues 9-12 in SNX-111 by the corresponding segment from SNX-183 (compound SNX-201) lowers the affinity for site 1 five-fold and increases the affinity for site 2 by a factor of 12. Conversely, introduction of the segment 9-12 from SNX-111 into the corresponding region of SNX-183 (compound SNX-202) increases the affinity for site thirty-four-fold and lowers the affinity for site 2 four-fold. Thus, a significant portion of the specific binding of the conopeptides to their receptor subtypes is due to the specific interaction of the middle segment with the receptor.

2. <u>Binding to Specific Proteins in Neuronal Tissue</u>

Conopeptide receptor polypeptides in rat brain or hippocampal synaptic membranes were chemically cross-linked to radio-iodinated SNX-111, SNX-183, or SNX-230 with a water-soluble carbodiimide, as detailed in Example 6. The radiolabeled peptides were separated by SDS-PAGE and visualized by autoradiography. These studies, as described below, provided further evidence that the two receptor sites identified by SNX-111 and SNX-230 are constituent parts of distinct calcium channels.

[^{125}I]-SNX-111, [^{125}I]-SNX-183, and [^{125}I]-SNX-230 were chemically crosslinked to synaptosomal membrane preparations and then subjected to SDS-PAGE followed by autoradiography. With [^{125}I]-SNX-111, essentially

Inhibition of incorporation of [^{125}I]-SNX-183 into the 210 kDa band by SNX-183 is consistent with the ability of this iodinated peptide to bind to both site 1 and site 2 with nearly equal affinity (Table 4). Thus, the displacement curve is shallow with an IC_{50} of 0.36 nM. Competition for the lower molecular weight bands was monotonic for both SNX-111 and SNX-183, and occurred only at higher concentrations of both peptides.

The crosslinking experiments strongly suggest that the conopeptide binding components of site 1 and site 2 are distinct molecular entities, both having M_r 210-220 kDa. Taken together, the binding studies, neurotransmitter release assays, and the crosslinking experiments indicate that site 1 and site 2 represent distinct calcium channels.

3. Binding to Distinct Regions of the Central Nervous System

It is now well established that subtypes of various neurotransmitter receptors are expressed in a tissue-specific and region-specific manner. The regional distribution of the OCT peptide binding sites in rat brain sections were determined by autoradiography, as detailed in Example 7. The results presented in Fig. 12 show that the distribution of binding of [^{125}I]-SNX-111 is highly localized (Figures 12A and 12B) and that non-specific binding is virtually non-existent (Figures 12C and 12D). Comparison of the specific binding of [^{125}I]-SNX-111 and [^{125}I]-SNX-183 (shown in Figures 12E and 12F) revealed overlapping but differential distributions of binding sites. Both ligands labeled the cortex, CA1, dentate gyrus, and caudate-putamen. In these regions, binding of [^{125}I]-SNX-183 was

The present invention provides a method and composition of the invention effective to reduce neuronal damage related to an ischemic condition in a human patient. The ischemic conditions may be due to an interruption in cerebral circulation, such as caused by cardiac failure, or other condition leading to global loss of blood supply to the brain, or to localized interruptions in blood flow, such as due to cerebral hemorrhage, or localized thrombotic or embolic events, or head trauma.

The ischemic condition which is to be treated using the method and composition is generally associated with stroke, defined as the sudden diminution or loss of neurological function caused by an obstruction or rupture of blood vessels in the brain, or by complete cessation of blood flow to brain, as in cardiac failure. In stroke, as well as in other types of cerebral ischemic conditions, the treatment method is aimed at preventing or reducing secondary brain damage resulting from the original ischemic event. The secondary damage typically includes cerebral cell destruction, or lesions, in the area surrounding the ischemic injury, in the case of focal ischemia, and also in areas of selective vulnerability, such as the hippocampus or basal ganglia, in the case of global ischemia. The secondary damage may often be manifested by functional impairment, such as loss of short-term or long-term memory. As will be seen below, the treatment method of the invention is effective in reducing or preventing both anatomical and functional secondary damage related to ischemia.

Pharmaceutical compositions include a neuronal-cell calcium channel antagonist compound having activities for selectively blocking norepinephrine

November 22, 1989, and August 2, 1990, respectively, and in related PCT application WO 91/079, the applicants have disclosed that omega-conotoxin peptides and related peptides which exhibit binding to and blockade of voltage-gated calcium channels are useful in reducing neuronal damage related to ischemic conditions. In the above-referenced applications, test compounds were administered at the time of or up to 1 hour following the experimentally induced occlusion which caused the ischemic event. As reported below, and according to an important feature of the invention, it has been found that there is little or no loss of protective effect of the neuroprotective compound when it is administered well after the ischemic event e.g., one hour following the period of transient occlusion. This delayed-administration protective event indicates that these compounds are effective in blocking the events leading from ischemic injury to secondary cerebral injury, since these events may occur over a period of many hours or even days after injury. Thus, the delayed administration may be effective to reduce secondary cerebral damage over a several hour period, or even a day or more, following the onset of ischemia.

The effectiveness of the composition in reducing neuronal damage related to ischemic injury has been examined in three animal systems which are widely employed as model systems for global ischemia and secondary stroke damage. The first system is the gerbil two vessel occlusion model of global ischemia produced by transient occlusion of carotid arteries of the neck. For clinical comparisons, the ischemia produced in this model has been likened to that produced by cardiac arrest, since all blood flow to

the applicants, published in PCT application WO 91/079.

A. Reduction in Anatomical Damage

1. Neuroprotective Activity of OCT Peptides. Ischemia in the gerbil model system was induced in anesthetized animals by occluding the two carotid arteries for eight minutes, as detailed in Example 8. OCT peptide was administered ICV during the occlusion period, or one hour following occlusion. Four to five days after occlusion and peptide treatment, the animals were examined histologically for anatomical damage in the hippocampal CA1 region, as detailed in Example 8.

Figures 10A and 10B are low-power micrographs of gerbil hippocampus CA1 region in animals after ischemia, and infusion of MVIIA OCT (SNX-111) (10A) or drug vehicle (10B). The arrows in the figures indicate the approximate borders of the CA1 region. At higher power, cells in the drug-treated ischemic animals appear normal (Figure 11A), whereas damage is apparent in the ischemic animals receiving vehicle alone (Figure 11B). Another example of complete drug protection is seen in Figure 11C, and an example of partial protection is seen in Figure 11D, where there is a small number of damaged cells.

Anatomical sections, such as those seen in Figures 10 and 11, were scored according to the criteria set out in Example 8. The extent of protection from ischemic damage in animals treated with neuroprotecting OCT peptides MVIIA and OCT GIVA was reported in PCT publication WO 91/079 for peptides administered prior to or 1 hour subsequent to ischemia.

In a second treatment method, OCT peptide was administered intravenously, as detailed in Example 9B. The degree of neuroprotection in global ischemia produced by SNX-111 administered 1 hour post-occlusion is indicated in Table 6. "NSD" in the table indicates that the value is "not statistically different" from the saline control value. In this study a dose of 15 mg/kg SNX-111 was effective to confer significant protection against cerebral damage subsequent to cerebral ischemia.

Table 6

Effect of intravenous administration of OCT MVIIA (SNX-111) 1 hour post-occlusion on hippocampal damage in rats

Treatment mg/kg	N	Mean Score	SEM	P
Saline	38	3.2	.14	-
1	12	2.9	.18	NSD
3	10	2.9	.28	NSD
5	9	2.4	.31	NSD
15	10	1.5	.28	P<.001

2. Neuroprotective Activity of OCT Peptides by Delayed Administration. Test compound was administered intravenously 6, 12, or 24 hours post-occlusion to a rat subjected to 4-VO as detailed in Example 9B. Results of a study in which saline, 1, or 5 mg/kg of OCT MVIIA was given as a bolus intravenously 6 hours post-occlusion are shown in Table 7. In contrast to administration 1 hour post-occlusion, when compound was given 6 hours post-occlusion, a significant reduction in neuronal damage was observed at the 5 mg/kg dose. Significant

in the compound when administered 6 hours post-occlusion.

The relationship between the time of treatment and neuroprotective efficacy was further investigated by comparing damage scores in animals receiving a single intravenous bolus injection of 5 mg/kg SNX-111 at 6, 12, 24, or 48 hours after the ischemic insult. SNX-111 provided highly significant neuroprotection ($p < 0.001$) whether administered at 6, 12, or 24 hours after reperfusion (Fig. 16). Damage scores were not significantly different between control animals and those receiving SNX-111 when vehicle or peptide was administered 48 hours after ischemia. *** Figure 17 shows that SNX-159, given at a bolus dose of 10 mg/kg 6 hours post ischemia, provides neuroprotection against forebrain ischemia. Shown in the bar graph is a comparison of the effects of 3 and 10 mg/kg SNX-159 and 3.5 mg/kg SNX-111 to saline treated animals.

Although the time at which neuronal damage is observed after temporary forebrain ischemia varies among neuronal populations, maximal damage is typically manifested within 72 hours after reperfusion (Pulsinelli, Kirino). To insure that administration of SNX-111 6h post-occlusion prevented, and not simply delayed, cell loss after the ischemic insult, hippocampal damage was compared in SNX-111 treated animals after 5 or 12 days of survival. Damage scores of SNX-111 treated animals were comparably reduced at both time points (Fig. 18), suggesting that SNX-111 was not merely postponing neuronal death.

B. <u>Functional Activity Protection</u>

IV. Neuroprotective OCT Peptide Compounds

A. Selection of OCT Peptides

Based on a sequence homology analysis of the peptides whose full sequences are known (Figure 1), the naturally occurring neuroprotective OCT peptides were grouped into distinct groups I and II, each with internal homologies distinct to that group, as can be appreciated from Figure 14. Group I includes active OCT peptides MVIIA (SNX-111) and MVIIB (SNX-159) which possesses a binding constant to the MVIIA site within the range of compounds with neuroprotective activity. Group II includes neuroprotective peptides GVIA (SNX-124), TVIA (SNX-185) and SNX-207. A third group includes inactive peptides SVIA (SNX-157), SVIB (SNX-183), SNX-230 and OCT peptides whose binding activities for the MVIIA site on neuronal membranes and/or activity in norepinephrine inhibition are outside the range of active compounds.

The three groups of OCT peptides are arranged in Figure 14 with their six Cys residues aligned, which places these residues at positions 1, 8, 15, 16, 20, and 28. To make this alignment, gaps were introduced at the positions shown in the three groups. Regions of sequence homology are indicated within R groups I and II by lower case letters a-f and s-v, respectively. In the analysis below, these gaps retain the assigned number shown in Figure 14, even though they represent amino acid deletions in the respective groups of active OCT peptides.

Sequence variation in the peptides, based on primary structure alone, was analyzed by adopting the following constraints:

1. The peptides in both active groups (I and II) include the Cys residues at position 1, 8, 15, 16, 20, and 28. Other Cys residues could be

allowed, including peptides in which the carboxy terminus is amidated or has a free acid form. Thus, the second group compounds include the peptide structures having the form: Region s (SEQ ID NO: 27)-X_1-Region t (SEQ ID NO: 31)-$X_2X_3X_4$-Region u (SEQ ID NO: 31)-$X_5X_6X_7$-Region v (SEQ ID NO: 27)-X_8-t, where X_1=K or L; X_2=X or R; X_3=T or L; X_4=S or M; X_5=T or S; X_6=K or R; X_7=R or K; and X_8=Y or R, and t= a carboxy or amidated carboxyterminal group.

5. Considering both active groups together, amino acid positions which are conserved in all active species are preserved. Thus, for example, the Cys residues, the 5-position glycine, the 13-position tyrosine, the 19-position serine, and the 26-position lysine are all preserved.

6. Considering both active groups together, there are amino acid positions which are likely to be variable within the range of active species. For example, the position 2 amino acid may be lysine or leucine, the position-3 amino acid may be glycine or serine, and the position 4 amino acid, hydroxyproline or arginine. In addition, if the two or more amino acids at a variant position are in a common substitution class, substitution within that class may be favorable. Standard substitution classes are the six classes based on common side chain properties and highest frequency of substitution in homologous proteins in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix (Dayhoff). These classes are Class I: Cys; Class II: Ser, Thr, Pro, 4Hyp, Ala, and Gly, representing small aliphatic side chains and OH-gr up side chains; Class III: Asn, Asp, Glu, and Gln, representing neutral and negatively charged side chains capable of forming hydrogen bonds; Class IV: His, Arg, and Lys,

corresponding to position 26 in the MVIIA structure shown in Figure 14. Since this substitution is at a conserved-sequence position, it is predicted that the neuroprotective activity would be lost or reduced. As discussed above, the SNX-195 peptide shows retention of MVIIA binding activity, but reduced norepinephrine release inhibitory activity, and weak neuroprotective activity compared with the unsubstituted MVIIA OCT.

As another example, the SNX-201 compound contains substitutions at positions 9-12 from Ser-Arg-Leu-Met to Arg-Lys-Thr-Ser, the sequence at positions 9-12 in the inactive SVIB OCT peptide. The position-9 substitution is not favored since Arg is present at this position in a non-neuroprotective compound, but not in one of the neuroprotective OCT peptides. The position-10 substitution is disfavored for the same reason. The position-11 substitution is favored, however, since the Leu to Thr substitution occurs within the neuroprotective peptides. The Met to Ser substitution at position 12 is favored for the same reason. Since the peptide modification contains two disfavored substitutions, it is predicted that the neuroprotective activity would be lost or reduced. As seen above, the SNX-201 peptide shows retention of MVIIA binding activity (Table 3), but reduced norepinephrine inhibitory activity (Table 2), and no neuroprotective activity at a concentration at which the unsubstituted MVIIA OCT/SNX-111 was found to be active (Figure 12).

B. OCT Peptides

The invention further includes the active OCT peptides formed according to amino acid selection rules 3 and 4 above, excluding the natural C-terminal

injury, such as stroke. The mechanism of neural protection by high-affinity OCT peptides presumably involves inhibition of voltage-gated calcium currents in neuronal membranes, thus blocking calcium influx into neuronal cells and the consequent release of neurotransmitters from the cells. This mechanism of OCT protection is consistent with the finding that neuronal damage in ischemia-related injury is associated with elevated intracellular calcium levels (Deshpande *et al.*).

In the present invention, it is further demonstrated that compounds which have a high selectivity ratio of binding for the OCT MVIIA binding site (site 1), in comparison to binding to OCT MVIIC binding sites (site 2) in neuronal tissue, as determined by above-described methods, correlate well with neuroprotective activity.

In practicing the screening method of the invention, compounds are tested for their binding affinities to OCT-MVIIA and OCT-MVIIC binding sites, as described above, by their ability to displace OCT MVIIA and OCT MVIIC, respectively, from synaptosomal preparations. Binding affinities for the two sites are determined, as described in Example 5. The binding affinities are compared to produce a selectivity ratio of binding to the MVIIA binding site using the formula:

$$\text{Selectivity ratio} = \frac{K_i(\text{MVIIA})}{K_i(\text{MVIIC})}$$

Representative ratios are shown in Table 4. The test compound is selected as a candidate for a neuroprotective agent if (i) the compound exhibits relatively high affinity binding to the MVIIA binding

OCT site 1 binding sites and to inhibit neuronal calcium currents to animals subjected to a cerebral ischemic event results in reduction of neuronal damage. Surprisingly, and in accordance with the present invention as described above, treatment with compounds of this class was shown to be effective, even when administration of the compound was delayed up to 24 hours following the onset of the ischemic event. This discovery has obvious implications and usefulness in the clinical setting, where time which elapses between an ischemic attack, such as a stroke, and diagnosis and treatment is typically at least several hours.

It is anticipated that pharmaceutical compositions containing compounds of the invention may be administered in any expedient formulation and route which results in delivery to the site of action, which is likely to be at or in close proximity to the ischemic region. Exemplary routes of administration are intracerebral and intravenous (bolus or slow infusion); however, it is appreciated that other routes of administration, including but not limited to intranasal, intrathecal, subcutaneous, or transcutaneous administration may be used in practicing the method of the invention.

The following examples illustrate, but in no way are intended to limit the present invention.

Example 1

Synthesis of ω-Conopeptide OCT MVIIC

OCT MVIIC was synthesized on a replumbed ABI model 430A peptide synthesizer, using standard t-butyloxycarbonyl (tBOC) chemistry, as described below. The synthesis was started from 0.4 mmole

Upon completion of the disulfide formation, the solution was acidified to pH ~3.5 with acetic acid, concentration under vacuum to ~15-20 ml, and gel-filtered on a Sephadex G-25 column (2.5 × 60 cm) eluting with 0.5 M AcOH. The pooled prepurified peptide fractions were further purified on a preparative HPLC column (Rainin Dynamax system, 4.14 × 30 cm, C-18 reversed phase packing material, 300 Å pore size, 12 μm particle size) using 0.1% TFA in water/0.1% TFA in acetonitrile gradient elution solvent system (40 ml/min pumping rate). The pure fractions were pooled, and lyophilized. The yield of purified peptide was usually 10-16% based on the loading capacity of the MBHA-resin.

Synthesis of other OCT peptides has been described in U.S. Patent No. 5,051,403, incorporated herein by reference.

Example 2

Calcium-Channel Antagonist Activity: Inhibition of Ionic Currents

Ionic currents through calcium channels were examined in cells that were voltage-clamped by a single patch-clamp electrode. These whole-cell patch-clamp studies were performed mainly on N1E115 mouse neuroblastoma cells, although a variety of cell types, including human neuroblastoma cell line IMR-32, have been examined.

A. Current Measurement Methods

Most measurements were obtained using a bath saline that allowed examination of the calcium currents in the absence of other ionic currents. These solutions contained 80 mM NMDG (as a sodium replacement), 30 mM TEACl (to block potassium

(typically -100 mV) to test potentials that ranged from -60 mV to +20 mV in 10 mV increments. The cell was held at the holding potential for 5 seconds between pulses. Protocols starting from other holding potentials usually covered the same range of test potentials.

B. Current Inhibition Measurement

Figure 3A shows calcium current traces from an N1E-115 mouse neuroblastoma cell. The figure is read from left to right in time, with downward deflections of the trace indicating positive current flowing into the cell. Currents were elicited by a voltage step from 100 mV to -10 mV. The cell was bathed in saline with sodium replaced by NMDG and 10 mM Ba^{++} instead of 2 mM Ca^{++}. Potassium currents were blocked by TEA in the bath and Cs^{+} in the pipet solution.

The three traces in Figure 3, labeled b-d, show decreasing calcium currents, with increasing MVIIA OCT peptide concentrations of 10 nM (b), 50 nM (c), and 200 nM (d).

The response of voltage-gated calcium current to increasing dosages of OCTs MVIIA and GVIA are shown in Figure 3B. The calculated IC_{50} is approximately 10 nM for GVIA and 100 nM for MVIIA. These values indicate extremely high specificity of the peptides for their site of action.

Table 1 compares IC_{50} values for GVIA, MVIIA, SVIB and SVIA OCTs. Whereas OCT GVIA and OCT MVIIA show 50% inhibition of the measured calcium current at nanomolar concentration range, IC_{50} values for OCT SVIB and OCT SVIA were not measurable within the range of concentrations tested, and are therefore listed as having IC_{50} values above the micromolar

of radioactivity. Radioactivity remaining in each slice was determined. Data were normalized to total cpm of radioactivity per slice: total radioactivity = S + B + slice, where S is the amount of radioactivity present in the stimulation buffer, and B is the amount of radioactivity present in the basal buffer. Stimulated release, as a percentage of total radioactivity = 100 (S/(S+B+slice)), and basal release, as a percentage of total radioactivity = 100 (B/(S+B+slice)). Concentration- effect graphs are plotted as in Figure 5A and Figure 5B. Computer aided curve fitting was used to determine IC_{50} values from such data.

Example 4

Synaptosomal Membrane Preparations

A. Mammalian-Brain Synaptosomes and Synaptosomal Membranes

Synaptosomes were prepared from rat whole brain or hippocampal region of brain. Rats were sacrificed, and forebrains were removed and transferred to 10 ml ice-cold 0.32 M sucrose containing the following protease inhibitors (PI): 1 mM EGTA; 1 mM EDTA; 1 uM pepstatin; 2 uM leupeptin. Brains were homogenized using a motor-driven Teflon-glass homogenizer (approx. 8 passes at 400 rpm). Homogenates from 4 brains were pooled and centrifuged at 900 xg for 10 minutes at 4 degrees. Supernatants were then centrifuged at 8,500 xg for 15 minutes. Resulting pellets were resuspended in 10 ml each ice-cold 0.32 M sucrose plus PI with vortex mixing. The suspension was then centrifuged at 8,500 xg for 15 minutes. Pellets were resuspended in 20 ml ice-cold 0.32 M sucrose plus PI. The suspension (5 ml/tube) was layered over a 4-step sucrose density gradient

reaction, the peptide solution was chromatographed by HPLC through a C-8 reversed phase column and eluted with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in water/acetonitrile (40:60 vol/vol). The major peak of radioactivity following the underivatized MVIIA OCT was collected.

The binding constant (K_d) for [^{125}I]-MVIIA OCT to rat brain synaptosomal membranes was determined by a saturation binding method in which increasing quantities of [^{125}I] MVIIA OCT were added to aliquots of a synaptosomal membrane preparation (10 ug membrane protein, suspended in binding buffer consisting of 20 mM HEPES, pH 7.0, 75 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 2μM leupeptin, .035 μg/ml aprotinin, and 0.1% bovine serum albumin (BSA), in a total volume of 0.5 ml). Binding at each concentration of labeled compound was determined in the absence and presence of 1 nM unlabeled MVIIA OCT to determine specific binding (as described in part B, below). The amount of labeled peptide specifically bound at each concentration was used to determine B_{max}, the concentration of specific binding sites on the synaptosomes, and K_d, following standard binding analysis methods (Bennett). Figure 6A shows a saturation binding curve of [^{125}I]MVIIA to rat synaptosomal membranes. Figure 6B shows a Scatchard transformation of the data, from which a calculated K_d of about 10 pM is determined.

B. Competitive Displacement Binding Assays

1. Binding of [^{125}I]-SNX-111 (MVIIA). Rat brain synaptosomal membranes prepared as described in Example 3 were suspended in a binding buffer

which is measured in the presence of excess unlabeled MVIIA OCT. Such values serve as approximations of the relative affinities of a series of compounds for a specific binding site.

The binding constant (K_i) for each test substance was calculated using non-linear, least-squares regression analysis (Bennett & Yamamura) of competitive binding data from 2 assays performed in duplicate on separate occasions. The relationship between K_i and IC_{50} (concentration at which 50% of labeled compound is displaced by test compound is expressed by the Cheng-Prusoff equation:

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where IC_{50} is the concentration of test substance required to reduce specific binding of labeled ligand by 50%; [L] is the concentration of [^{125}I]-MVIIA (SNX-111) OCT used in the experiment; and K_d is the binding constant determined for binding of [^{125}I]-MVIIA (SNX-111) OCT to rat brain synaptosomal membranes in saturation binding experiments. Table 3 summarizes computed IC_{50} for various OCT peptides for the MVIIA binding site of rat brain synaptosomal membranes.

2. <u>Binding of [^{125}I]-SNX-230 (MVIIC)</u>. Rat brain synaptosomal membranes were prepared as described above. OCT SVIB/SNX-230 was radiolabeled by iodination with ^{125}I-iodine by the Iodogen reaction, by standard procedures. Displacement binding of radiolabeled MVIIC on rat brain synaptosomal membranes was carried out as described for SNX-111, above. Results are shown in Figure 7B.

SDS-polyacrylamide gel. Gels were fix, stained with Coomassie blue, dried, and exposed to autoradiographic film by standard methods. For analysis of the concentration-dependent displacement of cross-linking in particular labeled polypeptide bands, two methods were used: 1) excision of the bands and direct counting of radioactivity in a gamma counter, or 2) densitometric analysis of the bands on the autoradiogram using a computer-aided imaging system and ^{125}I microscales (Amersham) as standards for determining film response curves.

Autoradiographs showing binding to separated proteins of [^{125}I]MVIIA/SNX-111 are shown in Figure 8A and Figure 8C. Binding of [^{125}I]SVIB/SNX-183 and of [^{125}I]MVIIC/SNX-230 are shown in Figures 8B and 8D, respectively.

Example 7

Localization of OCT Binding Proteins

A. Receptor Autoradiography

Whole brain was rapidly removed from male Sprague Dawley rats (250-350 mg.) and frozen in isopentane precooled on dry ice. The frozen brain was either used immediately or stored at -80°C and then used within three days. Coronal sections (20μM) were obtained at -15 to -20°C with a crystal microtome and thaw transferred to gelatin coated glass microscope slides. The sections were stored at -80°C and usually used within four weeks. The frozen tissue sections were allowed to dry at room temperature and then incubated with 200-250μl binding buffer (NaCl (75mM), EGTA (0.1mM), EDTA (0.1mM), leupeptin (2μM), aprotinin (0.5 unit/ml), bovine serum albumin (1.5%w/v), polylysine (MW=1000-4000, 1μM), and HEPES/NaOH (20mM, pH 7.5)) plus peptide for

techniques, both common carotid arteries were exposed, dissected free of surrounding tissue, and occluded with microvascular clamps approximately 3 to 4 mm above the clavicle. The occlusions were maintained for 8 minutes, timed while both arteries were occluded. There was generally a period of approximately 1 minute between clamping of each of the two arteries, and approximately 4 seconds between unclamping them. After the clamps were removed, the skin was sutured shut and anesthesia discontinued.

During or after the occlusion, an intracerebroventricular (ICV) injection aimed at the lateral ventricle was made. To accomplish this, a 10 microliter Hamilton syringe with a 27 gauge needle was filled with injectate by backloading to assure the absence of air in the system. A stiff plastic sleeve was slipped onto the needle so that 3.5 mm of the needle protruded past the sleeve. The skull around the bregma was exposed, a distance of 1.1 mm left of the midline was measured with a compass, and a distance of 0.4 mm posterior to bregma was approximated by eye. The needle tip was held perpendicular to the skull and inserted through it at that point by applying gentle pressure while twisting. It was advanced until the sleeve abutted the skull, and 5 microliters of injectate was infused over a period of approximately 3 sec. The skin was then sutured shut. Occluded animals received either drug or its vehicle. Injected, unoccluded controls were anesthetized, and received the ICV injection only.

Four to five days after the initial occlusion, animals were anesthetized with CO_2. The chest cavity was opened and the animal was perfused through the heart with approximately 3 milliliters of phosphate-

Sections, such as those seen in Figures 10 and 11, were viewed and scored by an investigator having no knowledge of the treatment of any particular sample. Ischemic damage was scored in the CA1 region of the hippocampus. Damage was generally seen as pink (eosinophilic) cytoplasm and shrunken, dark blue nuclei. Scoring was as described below:

Score	Observation
0	No damaged cells were apparent.
1.	Less than 25% damaged cells in a CA1 field, or damage was restricted only to the extreme edges of the CA1 region.
2.	Approximately 50% damaged cells in a CA1 field, or damage to less than half the length of CA1, but more than to only the extreme edges.
3.	Damaged cells outnumber normal cells to a maximum of 75%, with damage extending throughout most of CA1.
4.	Complete damage to CA1, with fewer than 25% normal cells surviving.

Example 9

Reduction in Anatomical Damage:

Global Ischemia Model 2

Global ischemic damage was examined in the rat brain model, employing the four-vessel occlusion method of Pulsinelli and Brierly (Pulsinelli) for introducing temporary global ischemia in rats. Although the two carotid arteries supply blood to the forebrain, their occlusion alone has only moderate effects on forebrain blood flow because the posterior

with a bulldog clamp. At the end of the 15 min. occlusion, the clamps were removed to allow reperfusion. An effective occlusion causes the animal to lose its righting response within about 1 min. of occlusion. If the animal did not lose the righting response or if it regained it during occlusion, the loops were pulled tighter to assure complete carotid occlusion. Animals that did not lose their righting response were eliminated from the study, because this suggested that there was still significant cerebral blood flow. This observation was confirmed by neuropathological analysis showing damage to be less in animals retaining their righting reflex than in animals that do lose their righting response. Some animals righted themselves once or twice during the occlusion but immediately lost the righting response again, and were not eliminated from the study. Any animal that righted itself and remained up was eliminated.

A. Intracerebroventricular Administration of OCT Peptide

Rats receiving intracerebroventricular (ICV) compound were anesthetized using halothane immediately following reperfusion, and compound contained in 5 μL saline or saline alone was injected into the lateral ventricle as for gerbils. The coordinates of the injection were 1.2 mm left of midline and 0.5 mm posterior to bregma, at a depth of 3-4 mm. Rectal temperature was monitored from just before occlusion, and for 4-6 hours post occlusion. Rats were maintained normothermic (rectal temperature at about 37 degrees) for 4-6 hours following occlusion, by means of a heating apparatus. The degree of

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

inhibition of N-type voltage-gated calcium currents, which is at least as great as that of an omega conotoxin selected from said group of omega conotoxins.

6. The method of claim 1, wherein the compound is further effective to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, as evidenced by a specific activity, in producing such inhibition of neurotransmitter release, which is at least as great as that of an omega conotoxin selected from said group of omega conotoxins.

7. The method of claim 1, wherein the compound is an omega conotoxin.

8. The method of claim 7, wherein the omega conotoxin is selected from the group consisting of OCT MVIIA, OCT MVIIB, OCT GVIA, OCT GVIIA, OCT RVIA, and SNX-207.

9. A method of screening compounds for use in reducing ischemia-related neuronal damage, such as produced by stroke, in a human, comprising

measuring binding affinities of the compounds to be screened to OCT MVIIA and OCT MVIIC binding sites in neuronal tissue,

determining from said binding affinities a selectivity ratio of binding for the MVIIA binding site,

selecting a compound if its selectivity ratio of binding for the MVIIA site is at least 100.

10. The method of claim 9, wherein the compound is selected if its selectivity ratio is at least 500.

carboxyterminal group, excluding the peptides in which X_2=X, X_3=T, and X_4=S.

16. A peptide having the structure SEQ ID NO: 20, wherein t = a carboxy or amidated carboxyterminal group.

```
                1         5         10        15        20        25        30

SVIA/
 SNX-    C R S S G S X C G V T S I - C C - G R C - - Y R G K - C T
 157

TVIA/
 SNX-    C L S X G S S C S X T S Y N C C R - S C N X Y S - R K C R
 185

SVIB/
 SNX-    C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C
 183

MVIIC/
 SNX-    C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
 230

 SNX-    C K G K G A X C R K T M Y D C C S G S C G R - R G K - C
 231
```

Fig. 1 (con't)

SVIB (SNX-183) C K L K G Q S C R K T S Y D C C S G S C G R S G K C NH_2

SNX-202 - - - - - - - - S R L M - - - - - - - - - - - - - - NH_2

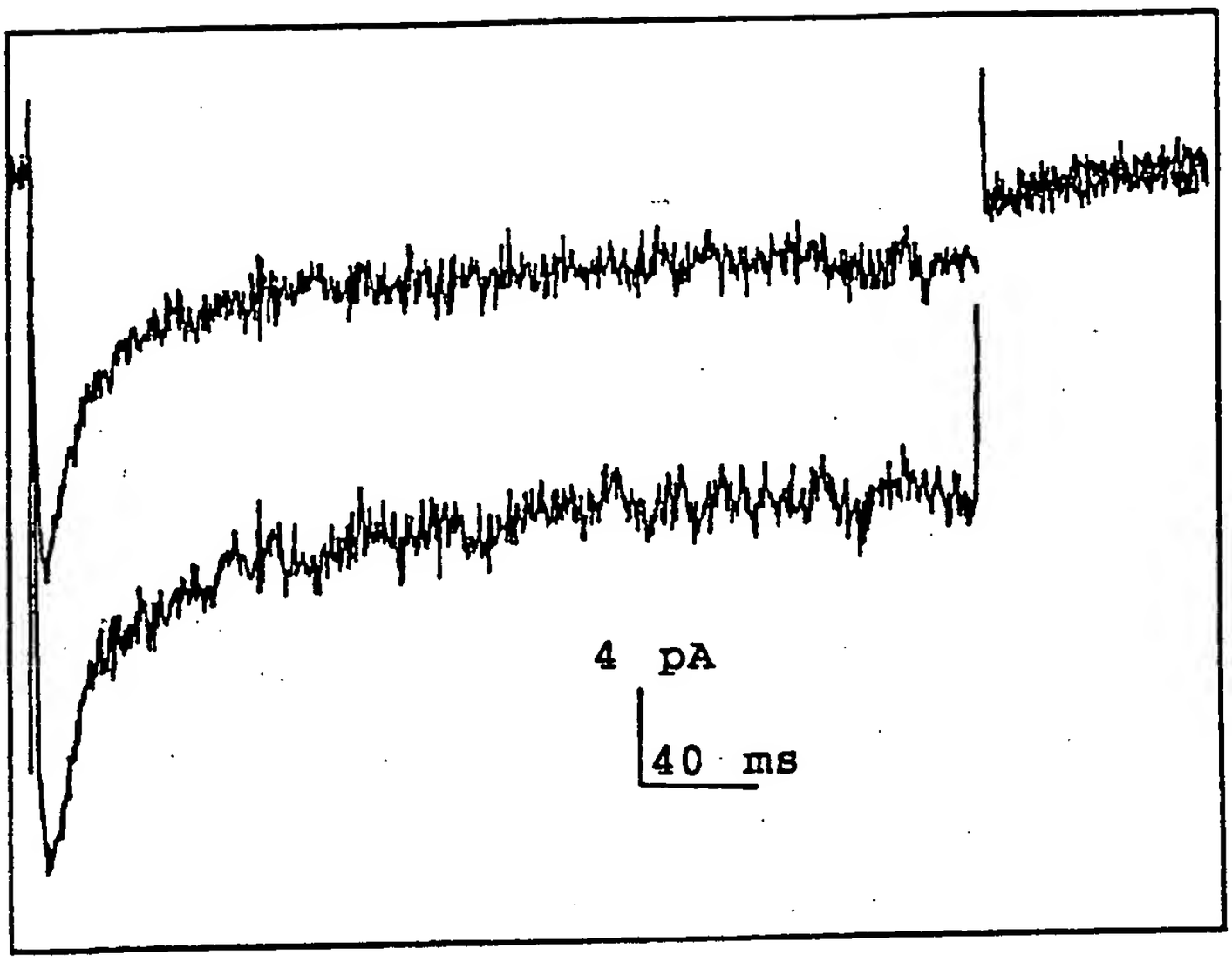
TVIA (SNX-185) C L S X G S S C S X T S Y N C C R S C N X Y S R K C R NH_2

SNX-207 - - - - - - - - - R L M - - - - - - - - - - - - - - - NH_2

SNX-236 - - - - - - - - - R L M - - - - - - - - P - - - - - - NH_2

Fig. 2 (con't)

Fig. 4A


4 pA
40 ms

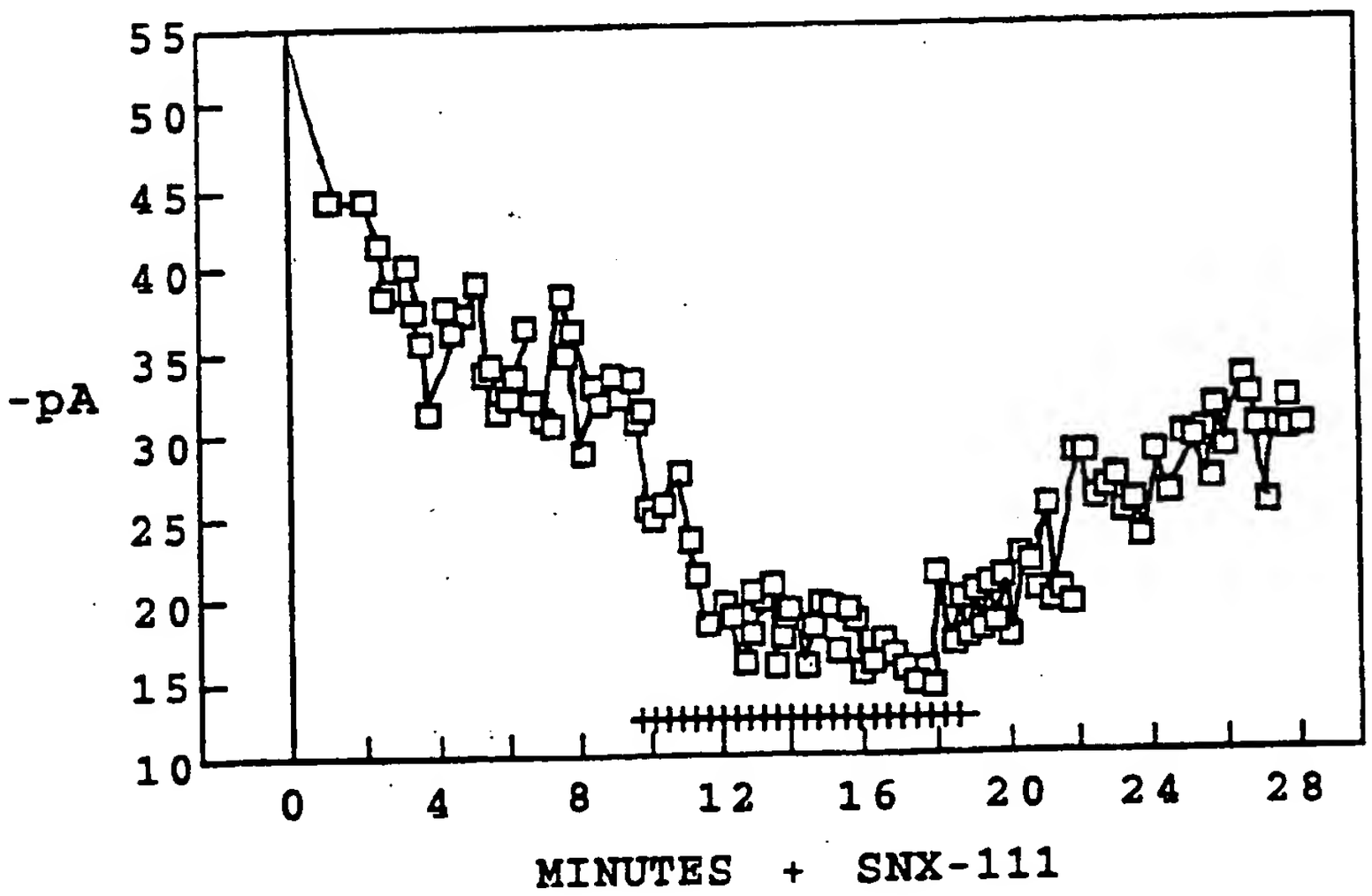

55
50
45
40
35
30
25
20
15
10
-pA
0
4
8
12
16
20
24
28
MINUTES + SNX-111

Fig. 4B

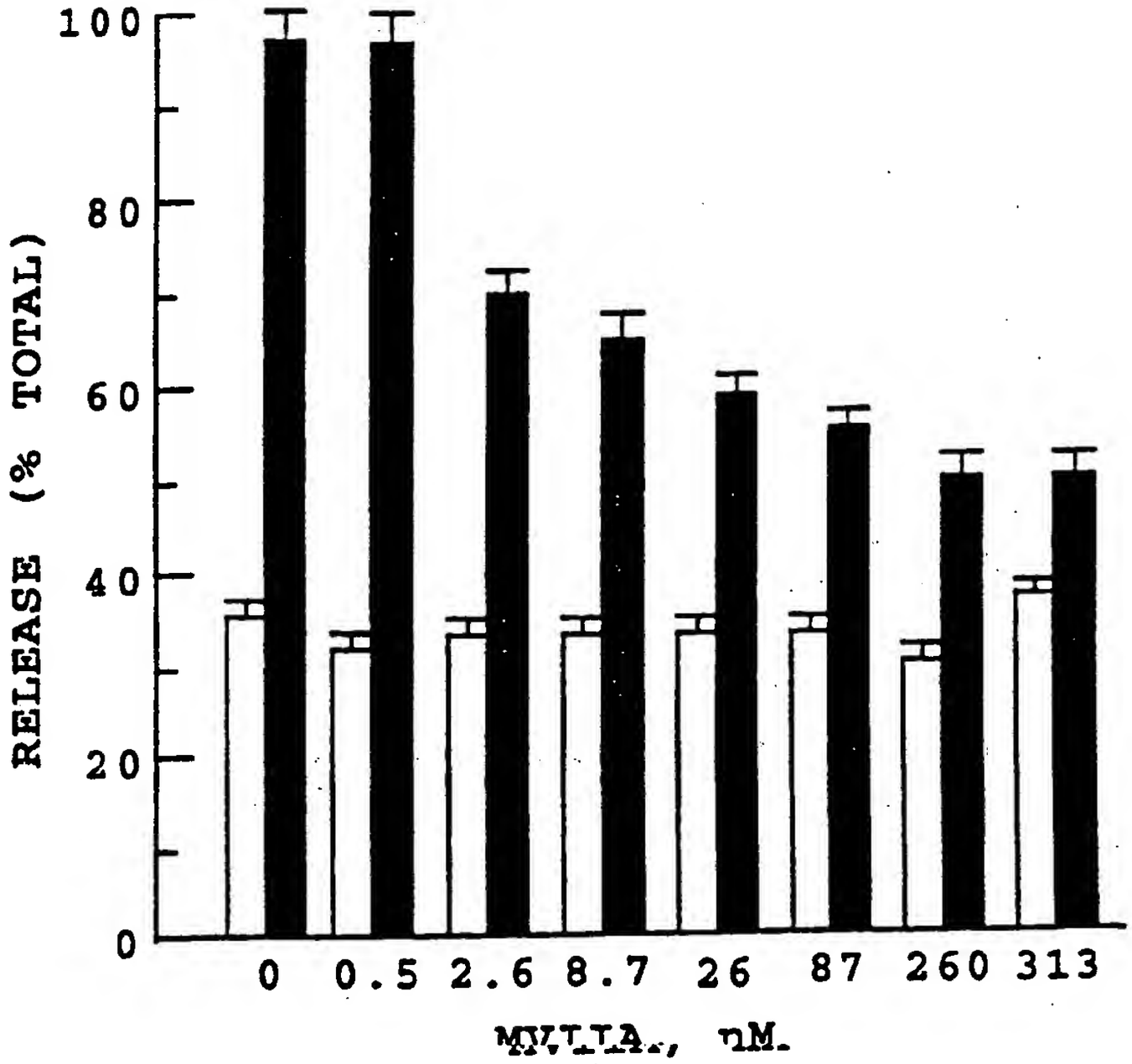

RELEASE (% TOTAL)
100
80
60
40
20
0
0 0.5 2.6 8.7 26 87 260 313
MYTLA., nM.

Fig. 5A

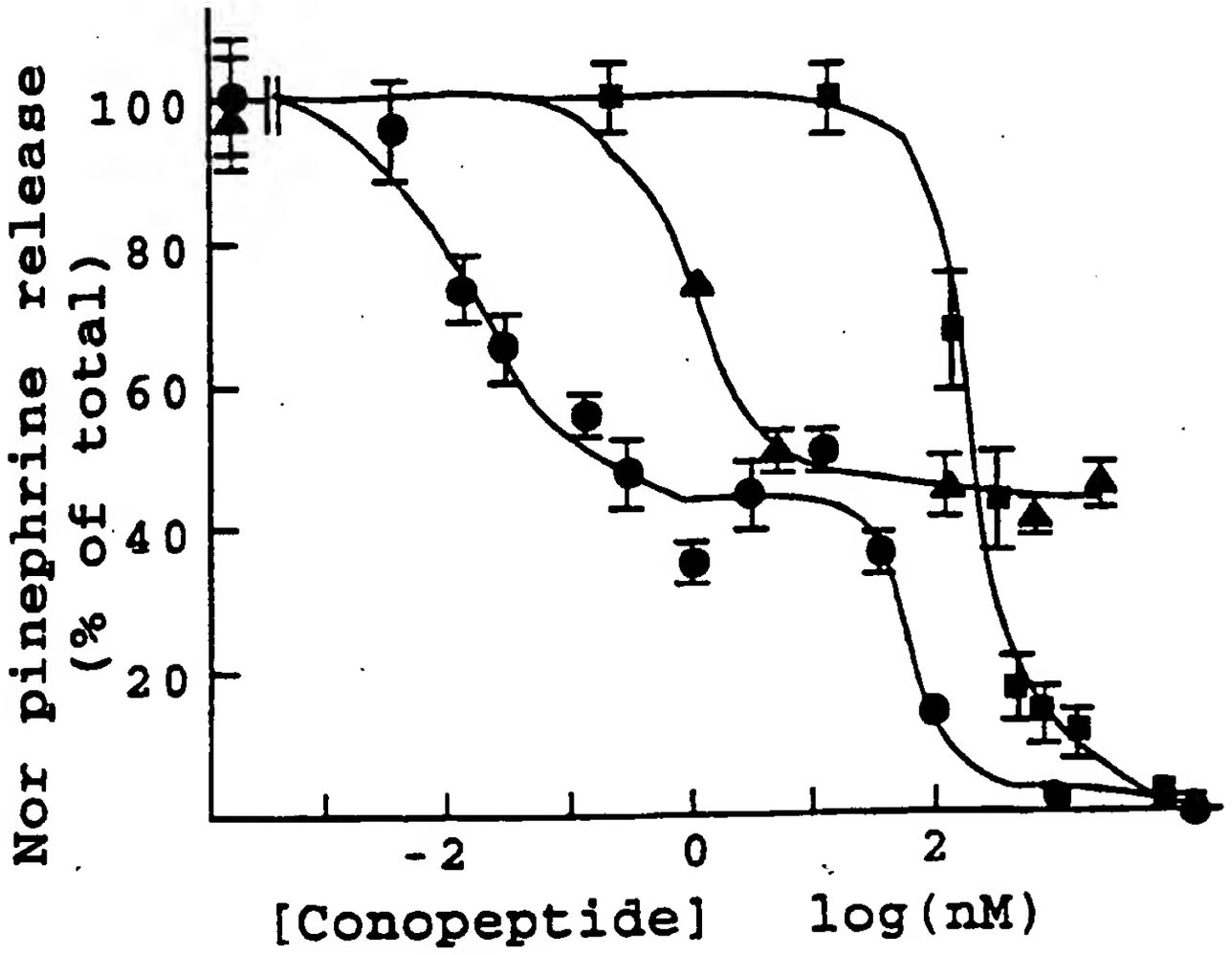

Nor pinephrine release
(% of total)
100
80
60
40
20
-2
0
2
[Conopeptide] log(nM)

Fig. 5B

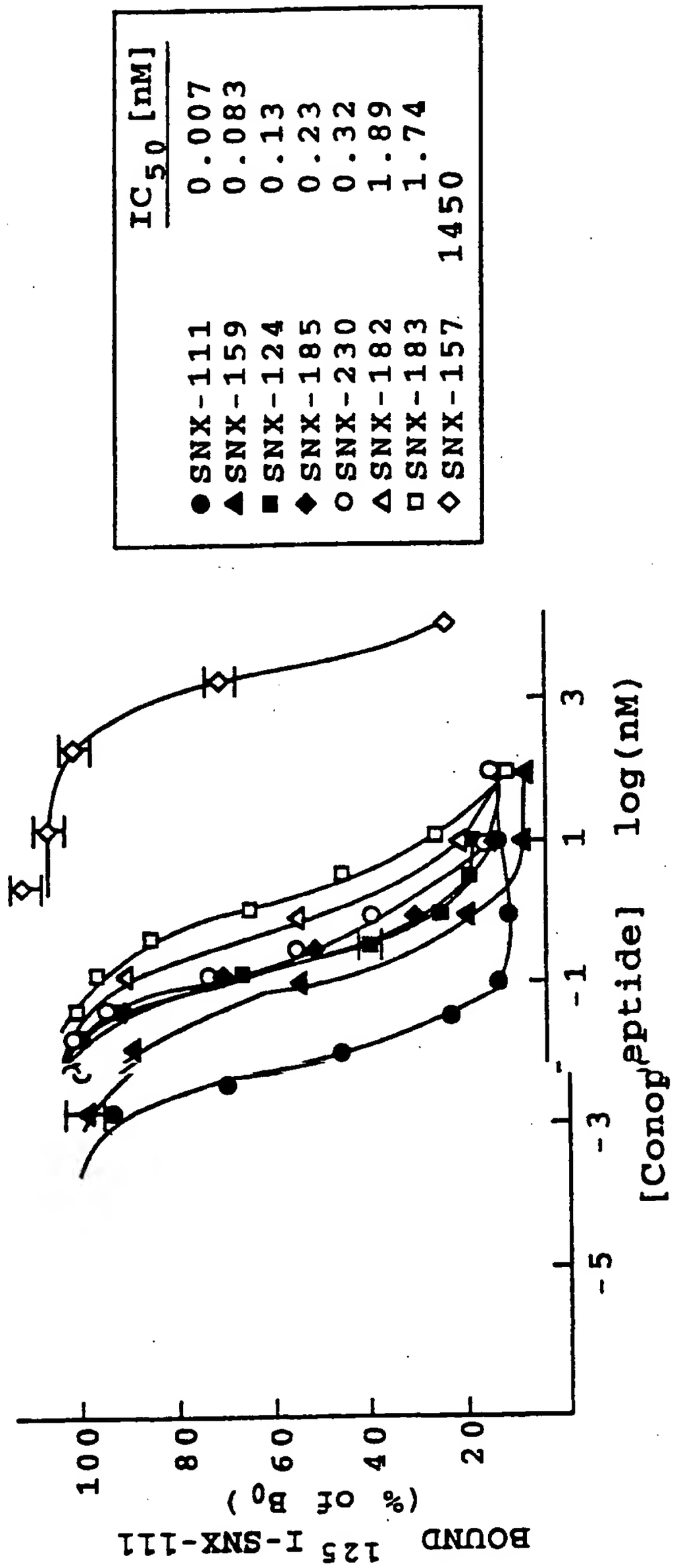

IC50 [nM]
SNX-111 0.007
SNX-159 0.083
SNX-124 0.13
SNX-185 0.23
SNX-230 0.32
SNX-182 1.89
SNX-183 1.74
SNX-157 1450
BOUND 125I-SNX-111 (% of B0)
100
80
60
40
20
-5
-3
-1
1
3
[Conopeptide] log(nM)

Fig. 7A

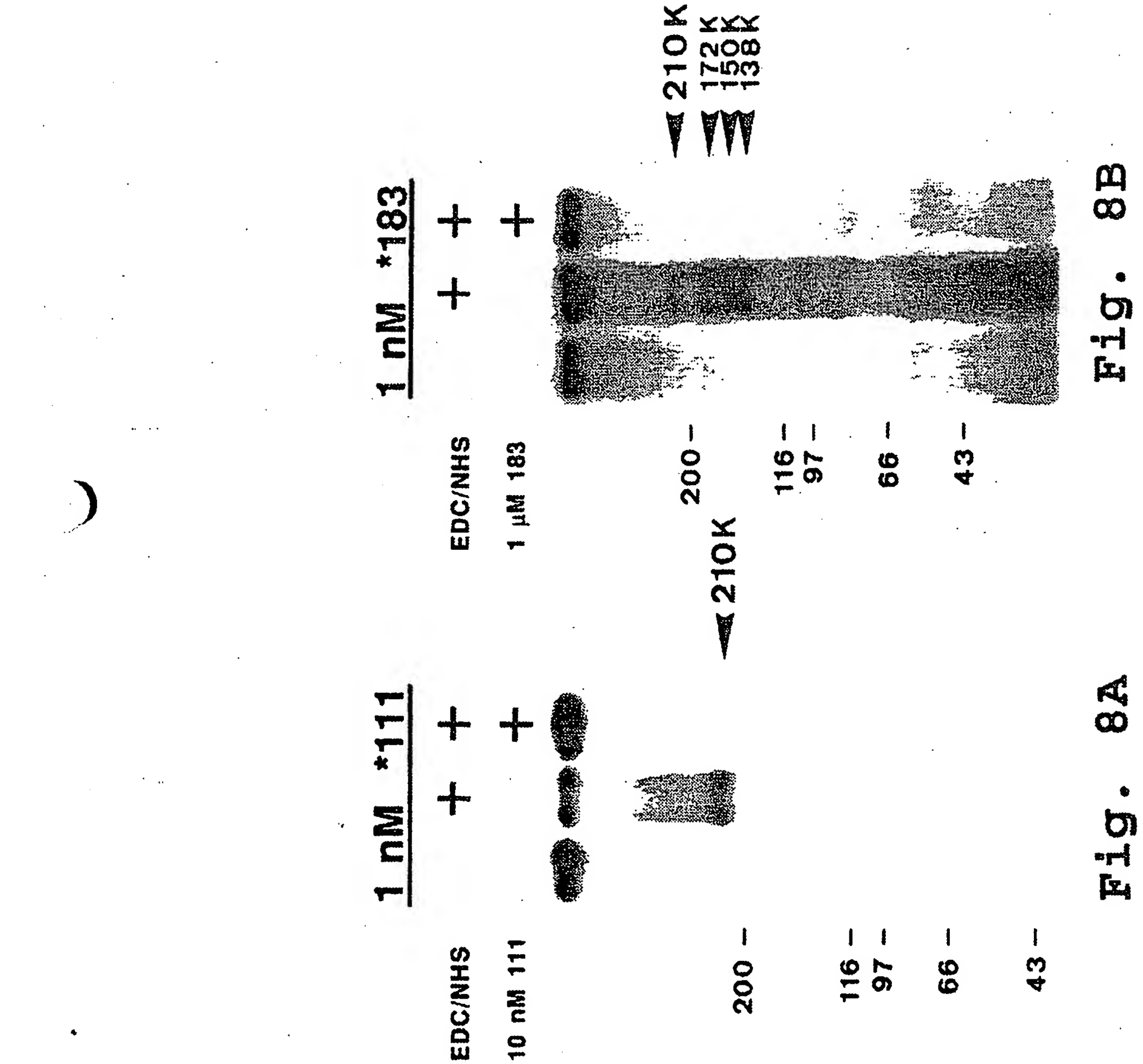

210K
172K
150K
138K
1 nM *183
+ +
+
EDC/NHS
1 μM 183
200 —
116 —
97 —
66 —
43 —
210K
1 nM *111
+ +
+
EDC/NHS
10 nM 111
200 —
116 —
97 —
66 —
43 —

Fig. 8B

Fig. 8A

Fig. 9A

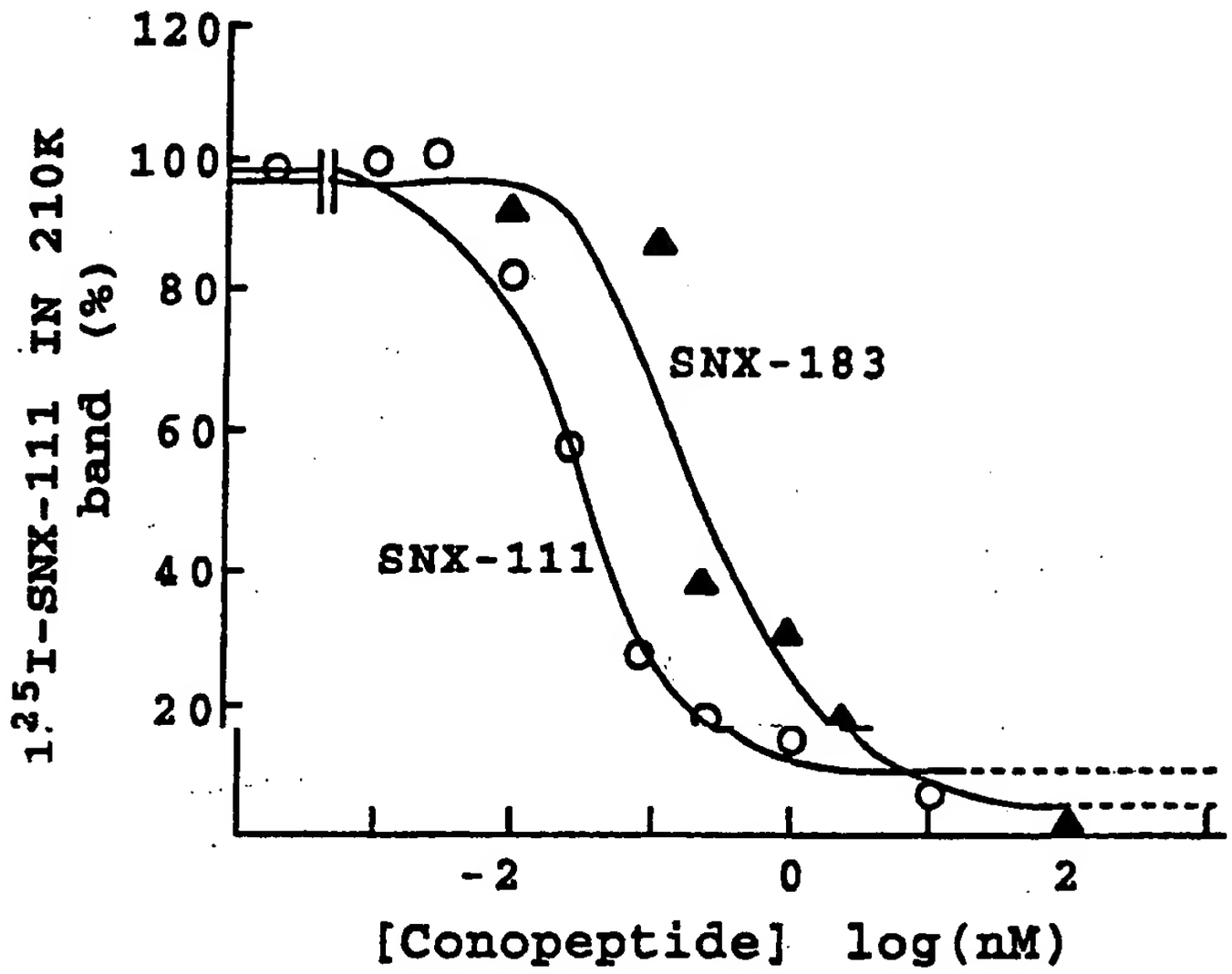
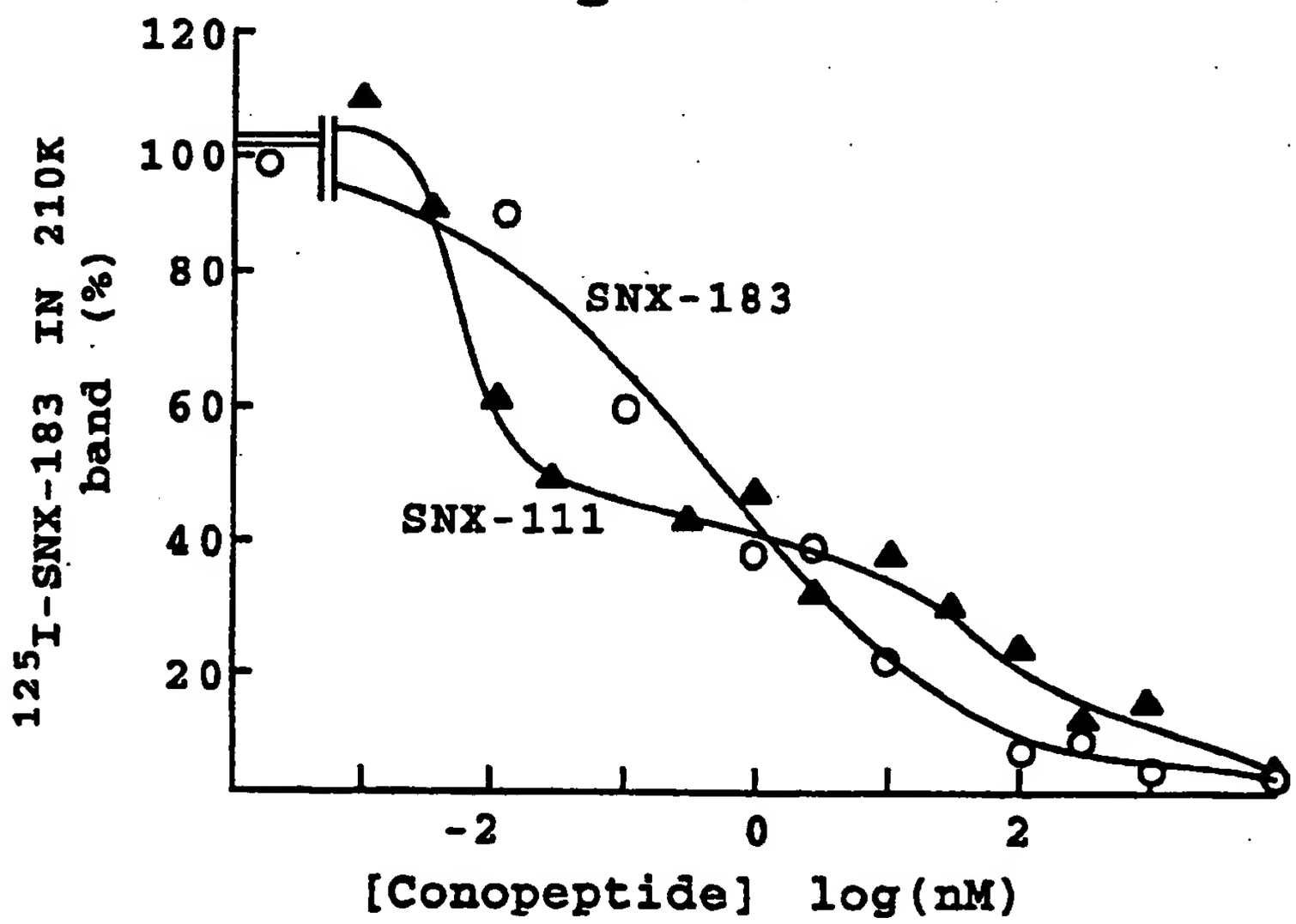

120
100
80
60
40
20
^{125}I-SNX-111 IN 210K band (%)
SNX-183
SNX-111
-2
0
2
[Conopeptide] log(nM)

Fig. 9B


120
100
80
60
40
20
^{125}I-SNX-183 IN 210K band (%)
SNX-183
SNX-111
-2
0
2
[Conopeptide] log(nM)

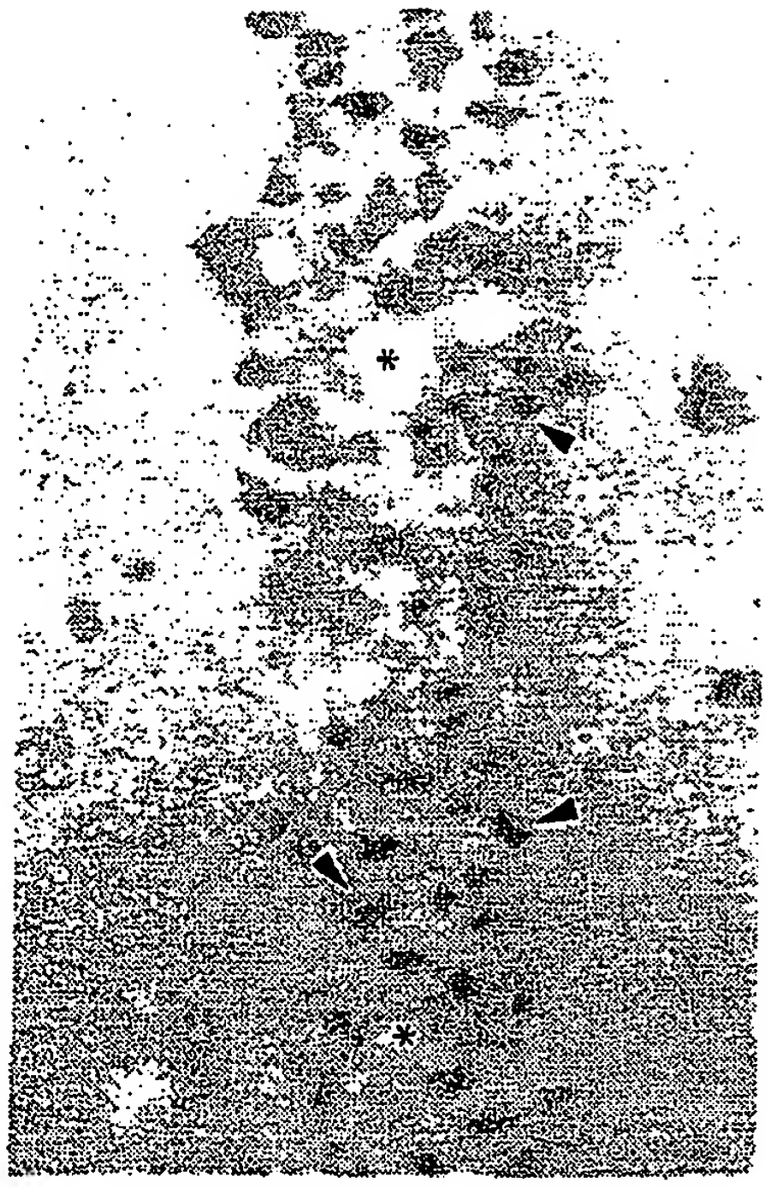


Fig. 11B

Fig. 11D

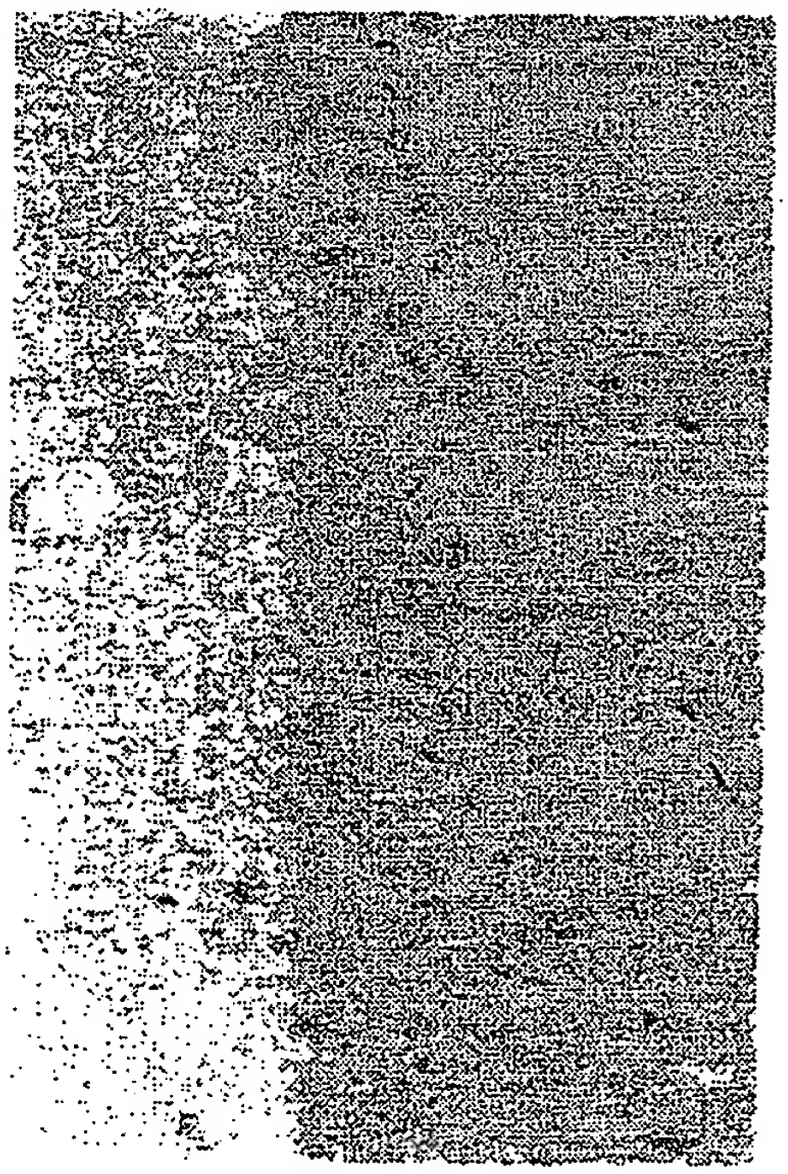


Fig. 11A

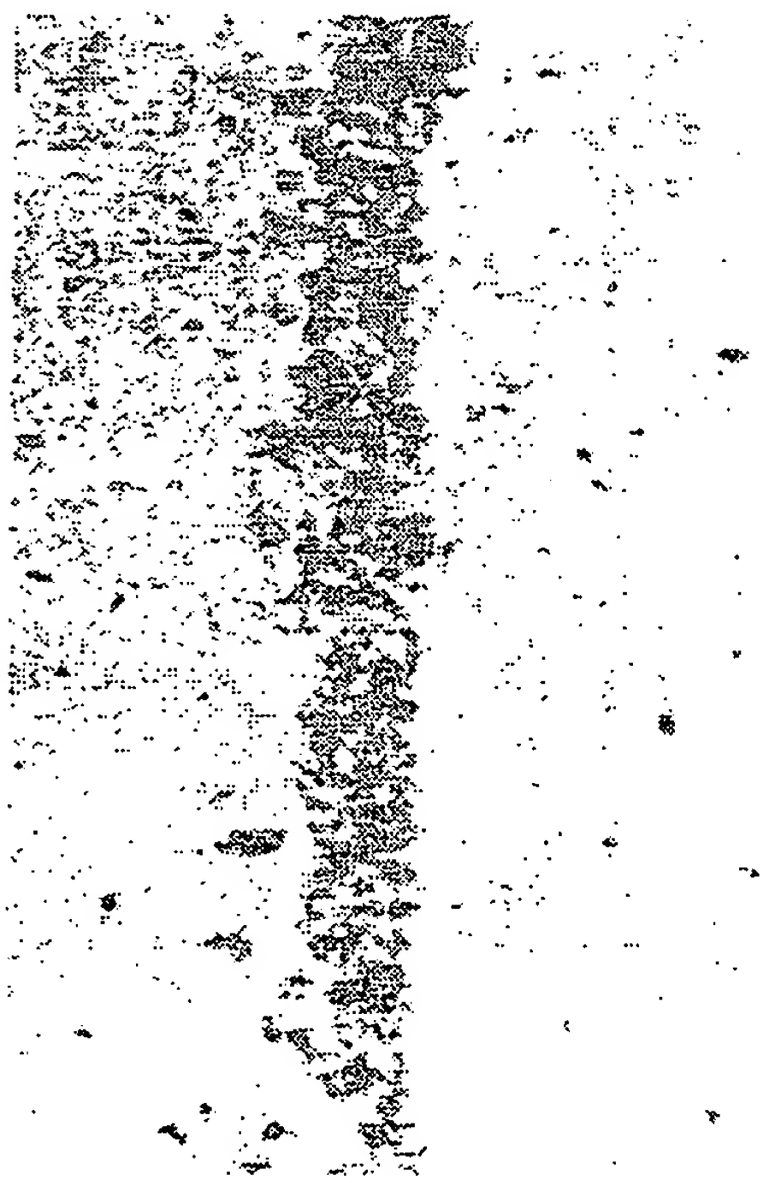


Fig. 11C

Fig. 12F

Fig. 12E

*183

Fig. 12H

*183 + excess 183

Fig. 12G

```
                1         5        10        15        20        25        30
                  a         b   c       d         e       f
I.
MVIIA     C K G K G A K C S R L M Y D C C T G S C - R - S G K - C
MVIIB     C K G K G A S C H R T S Y D C C T G S C N R - - G K - C

                s         t                   u                 v
II.
GVIA      C K S X G S S C S X T S Y N C C R - S C N X Y T - K R C - - Y
TVIA      C L S X G S S C S X T S Y N C C R - S C N X Y S R K - C R
SNX-      C L S X G S S C S R L M Y N C C R - S C N X Y S R K - C R
207

III.
RVIA      C K P X G S X C R V S S Y N C C S - S C K S Y - N K K C G
SVIA      C R S S G S X C G V T S I - C C - G R C - - Y R G K - C T
SVIB      C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C
GVIIA     C K S X G T X C S R G M R D C C T - S C L L Y S N K - C R R Y D
SNX-      C K S T G S S C S R L M Y N C C R - S C N T Y S R K - C R
SNX-      C K S T G S S C S R L M Y N C C R - S C N T Y S A K - C R
218

MVIIC/
SNX-      C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
230
```

Fig. 14

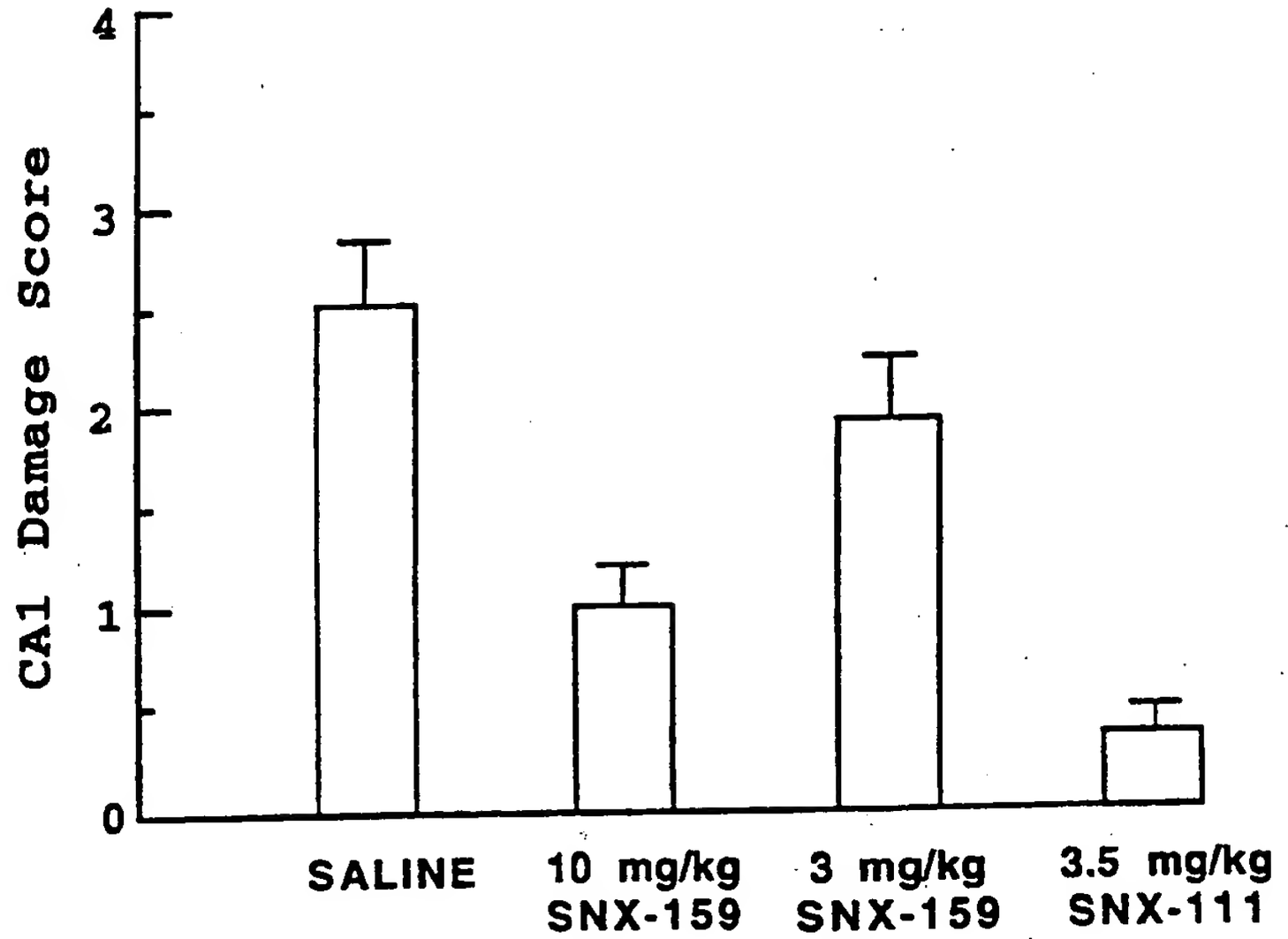

CA1 Damage Score
4
3
2
1
0
SALINE
10 mg/kg SNX-159
3 mg/kg SNX-159
3.5 mg/kg SNX-111

Fig. 17

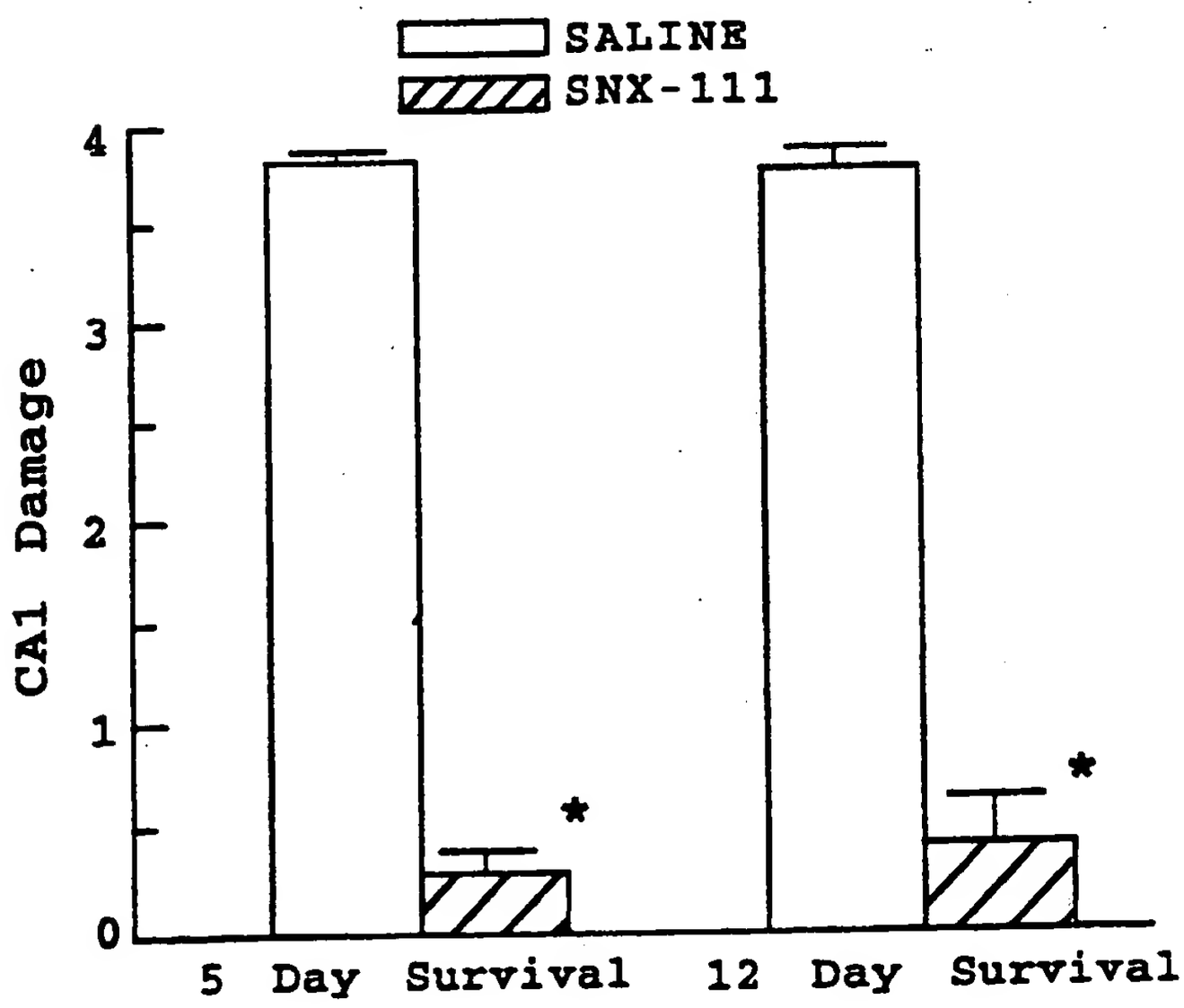

SALINE
SNX-111
CA1 Damage
4
3
2
1
0
*
*
5 Day Survival
12 Day Survival

Fig. 18

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 84, June 1987, WASHINGTON US pages 4327 - 4331 E.W. MCCLESKEY ET AL. 'omega-Conotoxin: Direct and persistent blockade of specific types of calcium channels in neurons but not muscle' cited in the application see discussion	3-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9209766
SA 67257

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/03/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9107980	13-06-91	US-A- 5051403	24-09-91
		AU-A- 6964091	26-06-91